THE REACTION RATE CONSTANT OF HYDRATED ELECTRON WITH SOME HEMOPROTEINS AS A FUNCTION OF THE pH

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1. Introduction

It has been found that for several proteins (gelatin [1], ribonuclease [1–3], lysosyme [1]) the reaction rate constant with the hydrated electron depends on the pH of the protein solution, during irradiation. This dependency has been attributed to several factors, such as change of total net charge of the molecule, change in reactivity of reactive groups upon dissociation and change of conformation. In our investigation of the reactivity of some hemoproteins with e^-_{aq} we have observed for metmyoglobin and methemoglobin a pH dependency of the reaction rate constant, that appears to be correlated with the net charge of the protein molecule. For cytochrome *c* a different behavior was found.

2. Materials

Methemoglobin (horse 2X cryst. 100%) and metmyoglobin (horse cryst.) preparations were obtained from Koch-Light Laboratories Ltd. and used without further purification. Pure monomer ferricytochrome *c* (horse heart) with an $A^{ox}_{550}/A^{red}_{280} = 1.30$ (Margoliash and Walasek [4]) was a gift of Mr. K.J.H. van Buuren, Laboratory of Biochemistry, B.C.P. Jansen Institute of the University of Amsterdam.

3. Methods

The reaction rate constants of e^-_{aq} with the investigated hemoproteins were measured with the pulse radiolysis technique joined with fast optical spectrometry, which has been described by others [5]. The hydrated electrons were produced by a pulsed beam of fast electrons from a 2 MeV Van de Graaff accelerator. The used pulse length was 25 nsec and the dose per pulse about 100–200 rad, leading to hydrated electron concentrations of about 0.2–0.4 μM. The protein concentration was 5–13 μM. The decay of the absorption of hydrated electron was measured at 700 nm. Under these conditions pseudo first order reaction kinetics were obtained. From the decay curve the value of the experimental reaction rate constant could be derived. The absorption of the investigated hemoproteins at that wavelength is negligible compared with the absorption of e^-_{aq}.

The concentration of the cytochrome *c* solutions was determined spectroscopically at 550 nm. The difference in molar absorption coefficient between oxidized and reduced cytochrome *c* was taken as 21.1×10^3 M^{-1} cm^{-1} [6]. The concentration of the methemoglobin and metmyoglobin solutions was determined after reduction with sodiumdithionite at 555 and 560 nm respectively with molar coefficients of 12.3×10^3 M^{-1} cm^{-1} and 13.0×10^3 M^{-1} cm^{-1} [7]. The molar absorption coefficient of hemoglobin is based on the heme-group.

For the measurements at different pH the following buffers were used:

Na_2HPO_4	$-KH_2PO_4$	3.3 mM	(pH = 5.6 – 8.0)
H_3BO_3	– NaOH	5 mM	(pH = 8.8 – 10.3)
Na_2CO_3	$-NaHCO_3$	5 mM	(pH = 9.9 – 10.3)
NaOH		1 mM	(pH = 11.2)

Oxygen which is an effective scavenger of hydrated electrons was removed by bubbling the solutions with argon (containing only 0.0003% oxygen) for about 45 minutes before the kinetic experiments. Hydrogen- and hydroxyl radicals which are also produced by the ionizing radiation, were scavenged by 2×10^{-3} M methanol. The experiments were all carried out at room temperature (19 ± 1°).

The experimental reaction rate constants were first corrected for the reactivity of the matrix solution and then for the effect of ionic strength with the Debye-Brönsted expression [5, 8]

$$\log (k/k_0) = 1.02\, Z_e Z_p \cdot \mu^{1/2} (1 + \alpha\mu^{1/2})^{-1}$$

in which α is 5.5 for metmyoglobin and cytochrome *c* and 6 for methemoglobin.

For the radii of the investigated molecules the following values were used:

cytochrome *c* 17Å [9], metmyoglobin 17.5 Å [10], methemoglobin 28Å [11], and e^-_{aq} 2.7 Å [12].

The effect on the reaction rate of the electrostatic interaction between the charge of the hydrated electron and the net charge on the protein surface (Debye factor) can be calculated with the well-known expression derived by Debye for the encounter rate between charged species [5, 8, 13]. The net charge of the protein molecules was derived from the titration curves of metmyoglobin [14], methemoglobin [15] and ferricytochrome *c* [16].

4. Results

For methemoglobin the experimentally found values after correction for the reactivity of the matrix solution are presented in fig. 1. From the curve through these points values were obtained for the reaction rate constant at some specific values of the pH. These reaction rate constants were corrected for the effect of ionic strength. Also at specific values of the pH the Debye factor was calculated.

The correction for buffer reactivity was usually about 10–20%. Only for the lowest and the highest pH's the correction was higher (20–50%). The correction for ionic strength was 0–300%.

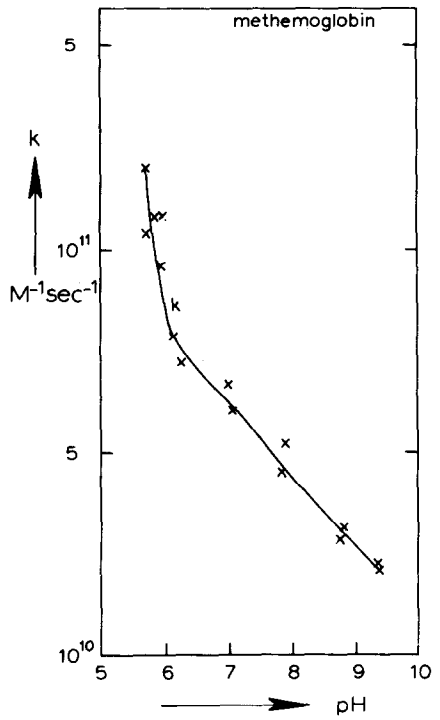

Fig. 1. The experimentally found values of the reaction rate constant *k* of methemoglobin with e^-_{aq} as a function of the pH after correction for the reactivity of the matrix solution. The methemoglobin concentration, based on the heme-group, was 13 μM.

The final values of the reaction rate constant and the Debye factor are presented in figs. 2–4 for methemoglobin, metmyoglobin and ferricytochrome *c*, respectively.

5. Discussion

For all hemoproteins the reaction rate constant within the pH range under investigation increases with decreasing pH. The Debye factor as plotted in figs. 2 and 3 for metmyoglobin and methemoglobin runs parallel to the plotted curves for the reaction rate constant. This suggests that the increase of reaction rate constant of these two hemoproteins between pH 9.5 and 6 is solely due to the increased attraction of the hydrated electron by the charges on the protein molecule.

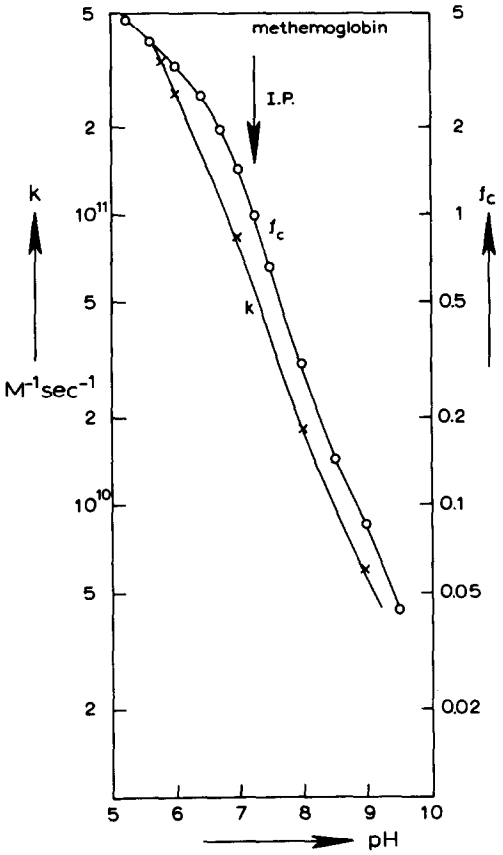

Fig. 2. The reaction rate constant k (X) and the Debye factor f_c (o) of e^-_{aq} with methemoglobin as a function of the pH. The methemoglobin concentration, based on the heme group, was 13 μM. The isoionic point is indicated by I.P.

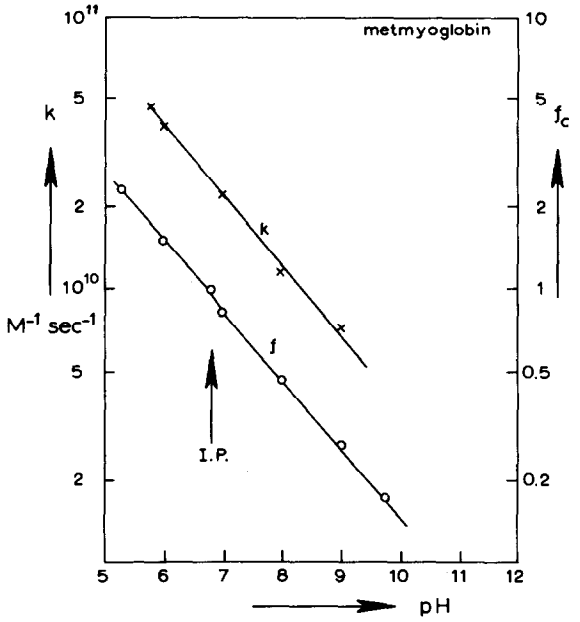

Fig. 3. The reaction rate constant k (X) and the Debye factor f_c (o) of e^-_{aq} with metmyoglobin (10 μM) as a function of the pH. The isoionic point is indicated by I.P.

For cytochrome c (fig. 4) in the pH range between 11–10 a similar behavior is found but between pH 10–8.5 the measured reaction rate constant increases more than could be expected on the base of electrostatic attraction only. Another strong increase of the reaction rate constant is found between pH 6.5–5.8.

At pH 8 the experimentally found rate constant of cytochrome c is about 2.9 times higher than the value that is obtained when the appropriate Debye factor is applied to the experimentally found value at the isoionic point (pH 10.03). At pH 6 this discrepancy has increased to a factor of about 3.6. The value of the rate constant at pH 7 is in good agreement with the value reported by Pecht and Faraggi [17].

In the pH range between 11–8.5 the charge increases mainly because of protonation of the lysyl residues that have their pK in this range. The lysyl residues appear to have a low reactivity with e^-_{aq} in both forms [18] so that their protonation does not lead to an increase in reactivity.

The further increase in the rate constant between pH 10 and 8.5 can therefore not be ascribed to either an increase in total charge or an increase in reactivity of amino acid side chains.

Land and Swallow [19] have also found a rise in the reaction rate constant of the hydrated electron with cytochrome c in the pH range 11–9.

An estimate of the reaction rate constant of cytochrome c on the base of its amino acid composition results in a value at pH 8.5 of about 3×10^9 M^{-1} sec^{-1}. The experimental value at pH 8.5 corrected for the net charge is 2.8×10^{10} M^{-1} sec^{-1}. This difference points to a large contribution of the heme-group to the reactivity of cytochrome c.

A change in reactivity of cytochrome c in the pH region 11–8.5 has been reported by Greenwood and Palmer [20] who found, that at pH 10 the rate of reduction with ascorbate and other reductants is

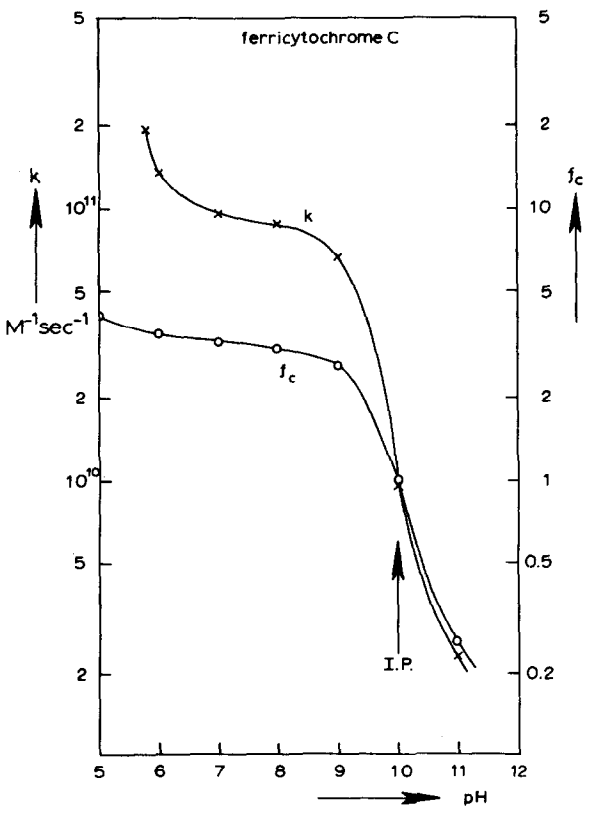

Fig. 4. The reaction rate constant k (×) and the Debye factor f_c (○) of e^-_{aq} with monomer horse heart cytochrome c (5 μM) as a function of the pH. The isoionic point is indicated by I.P.

much slower than in the neutral pH region. Land and Swallow [19] report a slow step in the reduction of ferricytochrome c with e^-_{aq} only at the alkaline side of neutrality. Several authors have reported evidence indicating that around pH 9.35 a change in interaction of some amino acid side chains in the environment of the heme-group with that group takes place [16, 20–24]. Sokolovsky, Aviram and Schejter [23] report that nitration of the tyrosyl-67 alters the environment of the heme-group. Some preliminary experiments on the reaction of e^-_{aq} with nitrated cytochrome c* show, that its reactivity in the pH region from 8.5–6.5 is about 50% of that of native cytochrome c in the same pH region. Apparently a change in the environment of the heme-group can result in a marked change in the reactivity of cytochrome c. Our results seem to indicate, that the heme-group is an essential part of the reactive site of cytochrome c and that the change in rate constant around pH 9.35 is caused by a change in reactivity of this site group.

In the pH range where the histidyl side chains change into the more reactive protonated form [18], a further increase of the reaction rate constant seems to be present in all 3 hemoproteins. At pH 6 this contribution to the reactivity is most evident in cytochrome c.

A more detailed analysis of these results will be presented after more data, e.g., on modified cytochrome c, have become available. Such work is in progress.

* Nitrocytochrome c was prepared by Mr. K.J.H. van Buuren following the method of Sokolovsky, Aviram and Schejter [23].

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